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^{13}C Nuclear Magnetic Resonance Spectra of Natural Undiluted Lipids: Docosahexaenoic-Rich Phospholipid and Triacylglycerol from Fish

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In the ^{13}C NMR spectra of natural, unenriched docosahexaenoic acid-rich sardine oil and cod muscle glycerophosphocholine significant signal intensity differences across the 1D spectrum between undiluted and diluted samples were observed. In undiluted samples ^{13}C−^{13}C 2D nuclear Overhauser enhancement spectroscopy (NOESY) interchain cross-peaks for CH, CH_2, CH_3, and C=C structures were observed. Results indicate that in undiluted natural lipids, NMR signal intensity is influenced by polarization transfer from the extended lipid structure. The NOE enhancement of specific molecular sites especially in unsaturated lipids is evidence that some natural lipids remain oriented relative to each other and in an orderly arrangement at the molecular level long enough for the effect to be detected by the NMR experiment. The presence of polyunsaturated fatty acids in mixtures of natural lipids could stabilize specific local molecular conformations within the remaining less saturated lipids.

KEYWORDS: **^{13}C−^{13}C 2D NOESY; ^{13}C NMR; polarization transfer; polyunsaturated lipids; fish oil; fish phospholipid; glycerophosphocholine; triacylglycerol**

INTRODUCTION

Natural triacylglycerols and phospholipids are high molecular weight viscous liquids. Closeness among the constituent lipid chains within triacylglycerols and phospholipids and between adjacent lipid molecules can result in two distinct distinguishable hydrophobic phases detectable by ^{13}C nuclear magnetic resonance (NMR) (*1*). In the absence of dilution with deuterated solvents, the ^{1}H frequencies in these lipids are poorly resolved overlapping peaks. Line broadening limits the ability to identify and assign sites close in space using two dimensional (2D) ^{1}H−^{1}H chemical exchange nuclear Overhauser enhancement spectroscopy (NOESY) experiments. Diluting lipid samples with solvent sharpens the proton spectrum but innately alters the relative distances between sites within and between lipid structures.

In binary mixtures of phospholipids with cholesterol, proton−proton broadening was dramatically improved and intramolecular cross-peaks were identified with 2D ^{1}H magic angle spinning NOESY techniques (*2*). Other NMR techniques including deuterium labeling of lipids can provide useful information (*3*). However, because the proton and deuterium sites are structurally and/or conformationally redundant, it cannot be determined which of multiple sites are close in space within a given sample (*4*). Two-dimensional heteronuclear ^{1}H−^{31}P techniques enable quantitative analysis of constituent phospholipids in membranes, but conformational information using heteronuclear NOESY is innately limited to short distances close to the phosphate headgroup (*5*).

The intensity of ^{13}C peaks in a one-dimensional (1D) experiment depends on polarization transfer from ^{1}H to ^{13}C atoms that are close in space—the NOE effect (*6*). For quantitative analysis, long recycle delays in highly diluted lipids are often used to minimize NOE-related intensity differences (*7*). Therefore, maximizing the NOE effect is a means whereby conformation and closeness in space within lipid structures can in principle be detected. The standard 2D NOE pulse sequence (*6*, *9*) using undiluted samples could enable detection of NOE-related changes in peak intensity because slowly relaxing sites are potential reservoirs of polarization that could be transferred to more rapidly relaxing sites. Thus, proton−proton−carbon NOE transfers occurring during the same time interval as relaxation would be detectable in appropriate 2D experiments; the product operators that define the 2D experiments are identical whether the spin $^{1}/_{2}$ nuclei is ^{1}H or ^{13}C (*8*). High-resolution ^{13}C spectra can be obtained in only 32 scans in undiluted lipid, which makes this approach both feasible and practical. Assignment of the sites at which proton−proton−

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carbon NOE interactions occur can provide molecular information on the uniformity and order of lipid packing.

Lipids rich in docosahexaenoic acid (DHA, 22:6*n*−3) are of broad interest due to the unique functions of DHA (*9*, *10*). Although chemically similar, *n*−3 docosapentaenoic acid (22:5*n*−3) cannot substitute for DHA in the brain or retina without sacrificing functionality (*11*). The conformation of DHA-rich lipids may be as important to functionality as the composition (*12*). NMR analysis of natural undiluted DHA-rich lipids is straightforward and may provide insight into lipid structure and conformation that are either not available or perturbed by solvent dilution. Natural triacylglycerols and phospholipids are preferred in these analyses to synthetic triacylglycerols and phospholipids because natural lipids are not structurally redundant. NMR experiments were performed to determine if specific molecular sites in undiluted natural lipids remain oriented relative to each other and in an orderly arrangement for long enough for an NOE effect to be observed. Specific lipid structural features that are more rigid could in principle be detected which affect the conformation of the remaining more mobile structural features in the lipids.

MATERIALS AND METHODS

Fish Oil. The fish oil utilized was purified distilled sardine oil containing 80 wt % *n*−3 polyunsaturated fatty acids (Max DHA, Jarrow Formulas, Los Angeles, CA). The fish oil was protected with 1% γ-tocopherol and 0.5% ascorbyl palmitate and remained encapsulated in brown gelatin, bottled, and refrigerated until use.

Preparation of Methyl Esters. The fatty acid methyl esters (FAME) were prepared by heating the lipid fractions with 4 mL of 15% acetyl chloride in methanol in a sealed vial at 70 °C for 3 h under nitrogen. The FAME composition of fish oil was determined using a Hewlett-Packard 5890 GC series gas chromatograph equipped with a 30 m × 0.25 mm i.d. DB-23 capillary column (J&W Scientific, Folsom, CA), automatic controller, autosampler, and HP 3396A integrator. Running temperature was from 170 to 210 °C at 5 °C/min; injection temperature was 250 °C. Beginning and ending hold times were both 1 min. Retention times for specific FAME were identified by comparison with FAME standards GLC mixture 68A (Nu-Chek Prep, Elysian, MN). Mono-, di-, and triacylglycerol and cholesterol percentages were estimated by separation with thin-layer chromatography (TLC) on 250 μm, 60 Å, silica gel plates (Whatman, Clifton, NJ).

Cod Muscle Phospholipids. The total lipid was extracted following the methods of Folch et al. (*13*) and Bligh et al. (*14*). Frozen cod fillets (368 g) were homogenized with 70 mL of 0.85% saline to a very fine paste in a food blender under nitrogen. The paste was transferred to a 2 L amber bottle. The residual material in the blender was washed with 30 mL of 0.85% saline and added to the paste; 736 mL of methanol with 0.01% butylated hydroxytoluene (BHT) was added to the cod paste followed by 736 mL of chloroform with 0.01% BHT under a stream of nitrogen. The mixture was stored at 4 °C for 12 h under nitrogen in the dark; 268 mL of 0.85% saline was then added under nitrogen and the mixture shaken vigorously for 5 min. The mixture was filtered through a Whatman no. 1 filter paper on a Büchner funnel with slight vacuum. The filter paper had been prewashed with chloroform/methanol (1:1). The residue on the filter paper was washed further with 100 mL of chloroform. The whole filtrate was transferred to a 2 L separation funnel for partitioning. The chloroform layer was then separated into a round-bottom flask and the solvent removed in a rotary evaporator under nitrogen. The dried whole lipid extract was dissolved in a small amount of chloroform/methanol (2:1 v/v, 0.01% BHT) and stored under nitrogen at −20 °C in the dark.

Column Chromatography for Separation of Phosphatidylethanolamine (PE) and Phosphatidylcholine (PC). The preparative column chromatography method of Christie (*15*) was followed. Thirty grams of whole lipid extract in 100 mL of chloroform/methanol (8.5:1.5 v/v, 0.01% BHT) was applied to a 50 cm × 60 mm i.d. glass column packed with 230−400 mesh silica gel 60 (Merck). This column was prepared by filling it with a slurry of 350 g of silica gel in chloroform. The solvents were flushed by nitrogen before elution, and the eluates were covered by nitrogen. The first solvent pass used 1400 mL of chloroform/methanol (8.5:1.5 v/v), the second solvent was 700 mL of chloroform/methanol (2:1 v/v), and last solvent was 3000 mL of methanol. The elution speed was ∼1−2 mL/min. The eluate collections were in 100−250 mL fractions and monitored by micro-TLC to assess the identity and purity of each eluate. The purified samples were dried under nitrogen and stored at −70 °C.

TLC Purification of PE and PC Eluates from the Column. Phosphoglyceride classes were separated by TLC on silica gel plates by the use of the developing solvents chloroform, methanol, and water (60:30:4 v/v, 0.01% BHT) in a nitrogen atmosphere. The bands for the choline and ethanolamine phosphoglycerides were detected by spraying with a methanolic solution of 2,7-dichlorofluorescein (0.01% w/v) and identified by the use of standards as reported previously (*16*).

Gas Chromatography (GC) of the Fatty Acids. FAME were separated by the same GLC procedure as in the fish oil samples.

NMR Analysis. NMR spectra were obtained on a Bruker QE 300 MHz spectrometer. Undiluted and diluted lipids were run in 5 mm amber tubes with broadband proton decoupling. For undiluted samples, the signal was locked on methylsulfoxide-d_6 placed in a glass capillary centered in the tube. All samples were run at 25 °C. Tubes were refrigerated in the dark when not in use, and all spectra on a given sample were obtained within 1 week. One-dimensional spectra were run with a standard pulse sequence for 4000 scans, with 2, 3.5, 5, and 20 s recycle delays. Spectra were processed with zero filling and exponential multiplication, with line broadening of 1.0.

The 2D pulse sequence was identical to ^{1}H 2D NOESY with τ_m defined as the mixing time (*17*). Two-dimensional spectra for neat samples were obtained using a 3.5 or 5 s delay between scans. Delays of up to 20 s were investigated to ensure that 3.5 s was adequate. Samples in $CDCl_3$ were obtained with a 3.5 s delay. Each 1D spectrum was the result of 64 scans containing 512 data points. The second domain contained 512 data points. Both domains were zero filled to 1024. Both frequency domains were at 75.6 MHz, with a spectral width of 13.9 kHz. Fish oil samples were run at 24, 40, 60, 120, 190, and 240 ms τ_m and cod muscle samples at 120, 150, and 300 ms τ_m. Sine bell squared multiplication of spectra was utilized in both dimensions. The 2D spectrum was processed in the magnitude mode so all cross-peaks have positive intensity.

For these relatively viscous lipid samples, ^{13}C inversion recovery experiments on fish oil were performed initially to define the time interval during which intersite polarization transfer was occurring. Inversion recovery delays were utilized from 10 μs to 600 ms to narrow the range of τ_m values used in the 2D spectrum.

RESULTS AND DISCUSSION

The fish oil sample was almost completely triacylglycerols. Less than 10% of the fish oil was monoacyglycerol and diacylglycerol. The cod muscle sample used was 99% glycerophosphocholine. Docosahexaenoic (22:6*n*−3), eicosapentaenoic (EPA, 20:5*n*−3), palmitic (16:0), and stearic (18:0) acids comprised the majority of fatty acids in both fish oil and cod muscle (**Table 1**).

1D ^{13}C Spectra. Fish oil and cod muscle were both run neat and in 50 vol % $CDCl_3$ under identical experimental and processing conditions. The high viscosity of the neat samples did not adversely affect signal-to-noise ratio. The peak positions for both neat and diluted samples agreed with previous results for DHA, EPA, and fish oil (*18*−*20*) and for natural salmon muscle lipid extract (*21*) with respect to the center peak of $CDCl_3$ (77.0 ppm). Gunstone (*18*) reported the carbonyl peak at 173.4 ppm for DHA. For both fish oil and cod muscle, the set of four DHA and EPA carbonyl peaks centered on 172.4 ppm, and the distinctive *n*−*3* polyunsaturated fatty acid (PUFA) −C̲H═ peaks for ω−3 and ω−4 (**Figures 1 and 2)** were in good agreement with previous results (*19*, *20*). However, it must

Table 1. Mole Fraction Fatty Acid Profiles in Sardine Fish Oil and Cod Muscle

fatty acid	sardine fish oil	cod muscle
14:0	5.1	—[a]
16:0	17.7	15.4
16:1	–	1.1
18:0	14.1	4.4
18:1*n*–7	–	2.1
18:1*n*–9	3.6	6.2
18:2*n*–6	–	0.9
20:1*n*–11	–	1.9
20:4*n*–6	–	2.5
20:5*n*–3	19.9	17.1
22:5*n*–3	–	1.0
22:6*n*–3	35.4	47.4
other	5.2	–
total	**100.0**	**100.0**

[a] Not detected or trace.

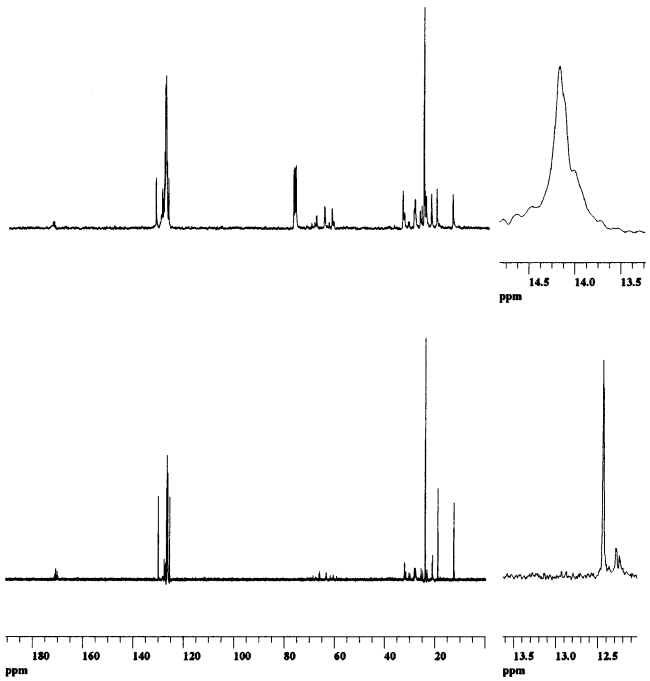

Figure 1. ^{13}C spectral intensity differences in fish oil with and without solvent with short (2 s) recycle time: (**A**, top) diluted with $CDCl_3$ (at 77 ppm); (**B**, bottom) undiluted. (Inset) Solvent-induced line broadening in the $-\underline{C}H_3$ region.

be kept in mind that spectroscopic frequencies change due to solvent effects.

The ^{13}C spectra (2 s recycle delay) of fish oil with solvent (**Figure 1A**) and without solvent (**Figure 1B**) show that changes in the relative intensities of peaks depends on chemical structure. The frequencies corresponding to the FA chain terminal $-\underline{C}H_2\underline{C}H_3$ structures (ca. 21 and 13 ppm) have line intensities more than twice as large in the neat sample as compared to the diluted sample. The intensity of two $-\underline{C}H=$ peaks at ca. 124 and 130 ppm compared to the other $-\underline{C}H=$ peaks are doubled in the neat sample. In contrast, the glyceryl peaks near 60 ppm are doubled in intensity in the *diluted* sample. Upon expansion of the range for $-\underline{C}H_3$ peak, in addition to a 2 ppm chemical shift, the line width is much sharper in the neat sample and resolution is improved. Two smaller $-\underline{C}H_3$ peaks ~0.2 ppm from the major $-\underline{C}H_3$ peak are observed in the undiluted sample, which are not resolved in the solvent-diluted sample due to the increased broadness of the $-\underline{C}H_3$ peak.

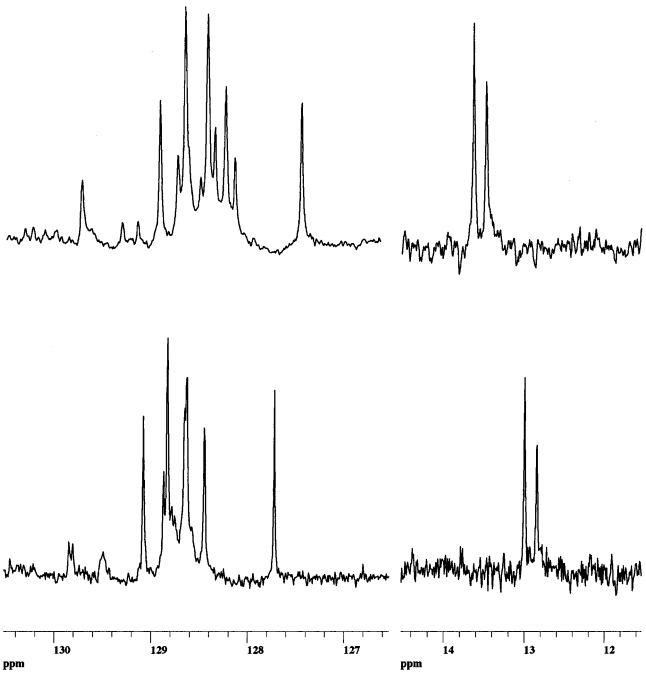

Figure 2. Expanded ^{13}C spectra of cod muscle glycerophosphocholine with and without solvent and long (20 s) recycle time: (**A**, top) diluted with $CDCl_3$; (**B**, bottom) undiluted.

Even with an order of magnitude longer recycle time (20 s), the relative intensities of peaks were found to depend on chemical structure. In the ^{13}C spectra of cod muscle with solvent (**Figure 2A**) and without solvent (**Figure 2B**), the corresponding $-\underline{C}H_3$ peaks are again sharper in the undiluted sample; the $-\underline{C}H=\underline{C}H-$ frequencies are also sharper, but fewer in number. Integration of the olefin range (126−133 ppm) yielded the same value for diluted and undiluted samples, indicating these changes are conformational.

2D ^{13}C Spectra. One-dimensional results indicated that 2D spectra of undiluted samples could yield novel information on lipid conformation not accessible in the presence of solvents. Two-dimensional experiments are polarization transfer experiments. The mixing time (τ_m) in the 2D $^{13}C-^{13}C$ NOESY experiment is the time interval during which polarization is built up and then transferred. Shorter τ_m values correspond to faster dynamic reaction, and longer τ_m values correspond to slower, longer lasting effects. Values of τ_m from 40 to 240 ms were used because shorter and longer τ_m values did not produce 2D cross-peaks. T_1 experiments with undiluted lipids also suggested inter- or intrasite polarization transfer was occurring during these time intervals at the sites that had the greatest 1D NOE enhancement. To avoid the problem of assigning magnetic site exchange among multiple redundant molecular structures, no attempt was made to interpret interactions in the aliphatic carbon (~22−35 ppm) or carbonyl (~165−180 ppm) regions.

The $-\underline{C}H_2\underline{C}H_3$ frequencies (ca. 21 and 13 ppm) at the terminal end of all the FA chains were nearly identical in all of our runs. Fish oil and cod muscle exhibited strong 2D cross-peaks among the $-\underline{C}H_3$ groups (~10−20 ppm) (**Figures 4 and 5**) and among carbons in the $-\underline{C}H=\underline{C}H$ region (~126−132 ppm) (**Figures 3 and 6**). In the $-\underline{C}H=\underline{C}H-$ region (**Figure 3**), the fish oil 2D intensity contour pattern changed from symmetrical at shorter τ_m values (up to 120 ms) to progressively more asymmetrical as the mixing time increased. There were generally no cross-peaks involving the glycerol, phosphocholine, or carbonyl moieties. For cod muscle (**Figure 6**) at τ_m = 120

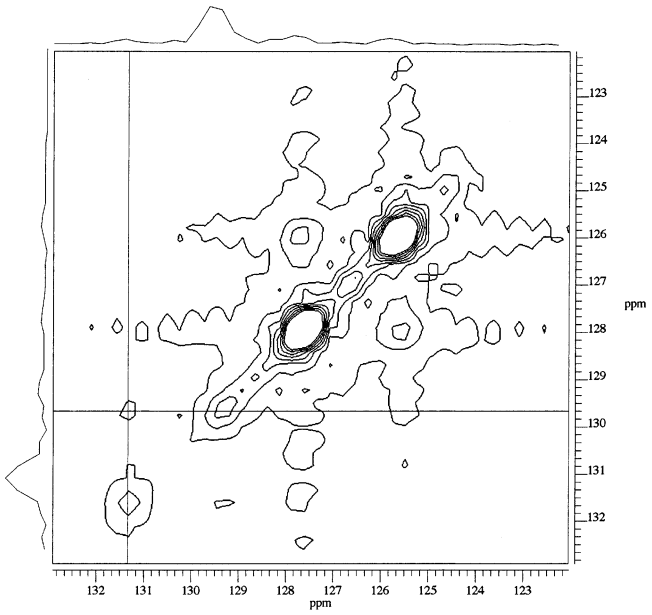

Figure 3. –C̲H═ region of ^{13}C–^{13}C 2D NOESY spectrum of undiluted fish oil (τ_m = 120 ms).

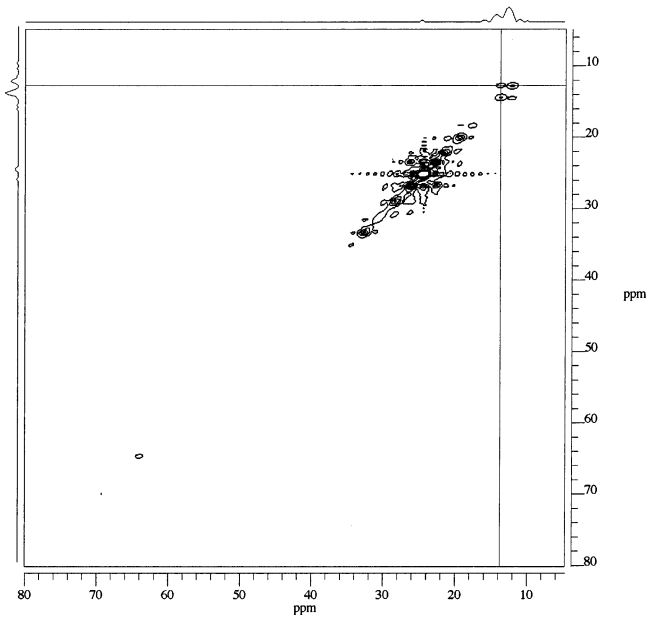

Figure 4. Aliphatic region of ^{13}C–^{13}C 2D NOESY spectrum of undiluted fish oil (τ_m = 190 ms).

and 150 ms, the glycerol and phosphocholine peaks were not observed even on the diagonal of the spectrum.

At τ_m = 40, 120, 190, and 240 ms, fish oil exhibited cross-peaks for two terminal −C̲H$_3$ groups. Fish oil cross-peaks were most intense at 120 ms (**Figure 4**). At 240 ms, the −C̲H$_3$ cross-peaks were less intense but still visible. At shorter τ_m (i.e., 24 and 40 ms) the cross-peaks were weak or not observed; however, four signals were observed in the methyl region (10−18 ppm) instead of two (**Figure 5**). An attached proton test was used to confirm that signals observed below 22 ppm were exclusively from −C̲H$_3$. Within the set of four signals, the two with greatest intensities corresponded to the two −C̲H$_3$ signals seen at longer mixing times. The intensities of the two main −C̲H$_3$ signals were nearly equal in 2D fish oil and cod muscle spectra (unlike the 1D results). This is consistent with magnetic site exchange between two methyl group conformational states.

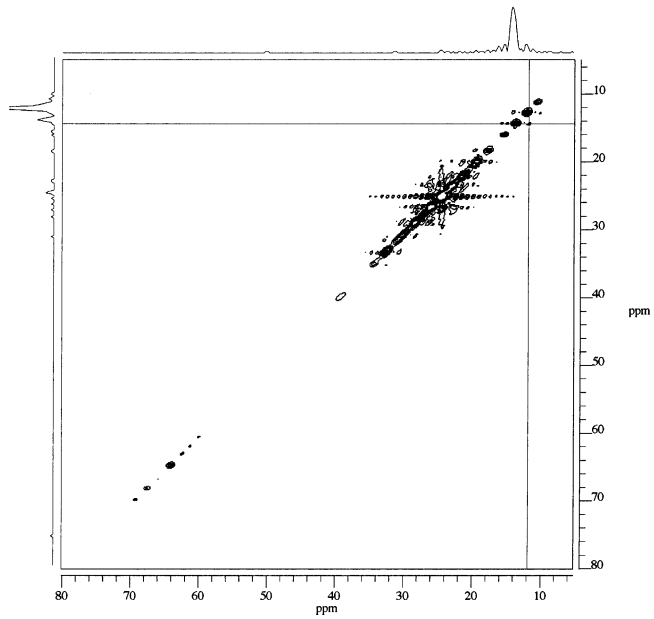

Figure 5. Aliphatic region of ^{13}C–^{13}C 2D NOESY spectrum of undiluted fish oil (τ_m = 40 ms).

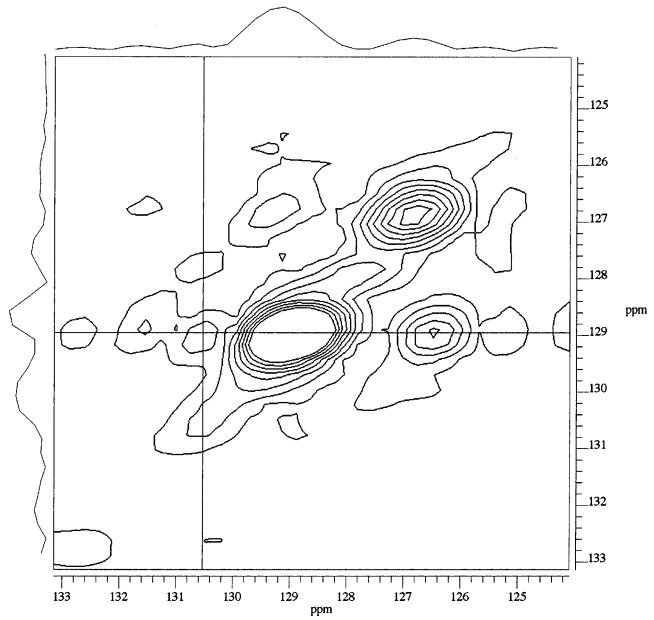

Figure 6. –C̲H═ region of ^{13}C–^{13}C 2D NOESY spectrum of undiluted cod muscle glycerophosphocholine (τ_m = 150 ms).

The samples with 50 vol % $CDCl_3$ were run and processed identically to the undiluted samples. At τ_m = 120 ms, the diluted samples did not show resolvable 2D ^{13}C−^{13}C cross-peaks, with the possible exception of one set of cross-peaks in the −C̲H═C̲H− region. Addition of solvent in 2D runs significantly reduced the overall signal-to-noise ratio. Specifically, 2D experiments on samples with $CDCl_3$ showed dramatic reductions in the −C̲H$_3$ signal intensity as compared to undiluted samples. Because chemical shift and magnitude perturbations from the solvent(s) are comparable to changes in chemical shift and magnitude due to structural differences among the lipid components, removing solvents can enhance information on conformational interactions between lipid molecules.

Undiluted natural lipids can be considered as strongly concentrated triacylglycerol or phospholipid solutions. The 2D ^{13}C−^{13}C NOESY experiments were conducted to identify

the portions of these structures that are closest in space and/or most similar in orientation among and between lipid chains. Solvents were assumed simply to push lipids further apart, precluding identification of sites where lipids are structurally or conformationally closest in space. Using undiluted lipids may maximize the ability to detect potential intra- and intermolecular NOE effects.

The NOE effect on a ^{13}C peak intensity is normally limited to the carbon atom to which the proton is chemically bound. In undiluted lipids, the proton frequencies are broad features but the carbon frequencies are very sharp. The broad proton peaks are from closeness in space and/or magnetic site exchange between conformationally related protons (*22*). Thus, each ^{13}C peak intensity in undiluted lipids includes an additional NOE component from the magnetic site exchange from each proton chemically bonded to the carbon. In diluted samples, the chemical environment instead is the deuterated solvent that does not contribute to the NOE and/or proton magnetic site exchange taking place among lipid molecules. Thus, an unequal distribution of this NOE effect among the structural components in the lipids should correspondingly yield novel spectroscopic information detectable in the appropriate ^{13}C experiment. Recently, a 2D experiment using partially ordered solid samples successfully converted broad 1D peaks into sharp peaks in the second dimension (*23*). We propose that undiluted viscous heterogeneous lipids remain partially ordered liquids and that this explains both the sharp ^{13}C and the broadened ^{1}H spectra.

The $^{13}C-^{13}C$ 2D NMR NOESY experiments used the broadened proton frequencies to produce sharp cross-peaks in the second dimension, that is, identifying the ^{13}C frequencies that simultaneously share the same broadened proton peaks. The 2D pulse sequence to detect NOESY closeness in space during τ_m is identical with that used to detect magnetic site exchange (*23*). Thus, closeness in space and/or magnetization transfer is occurring between the somewhat less ordered (more mobile) and the somewhat more ordered (less mobile) components in the lipid chains. We hypothesize that magnetic site exchange is occurring among inter- and intramolecular sites on structurally similar heterogeneous lipid chains during the 2D experiment. Each fatty acid in each triacylglycerol and phospholipid molecule in fish oil and cod muscle has a terminal $-\underline{C}H_2\underline{C}H_3$ group that has a nearly identical value of chemical shift, but with slightly different mobility. With an increasing τ_m value, polarization from some of the slightly faster moving $-\underline{C}H_3$ peaks is transferred to some of the slightly slower moving $-\underline{C}H_3$ peaks on other lipids. For this to be observable in the NMR experiments, naturally heterogeneous lipids must have an unexpected degree of uniformity in lipid conformation and mobility, despite their rather complicated FA composition profile.

In the $-\underline{C}H=$ region, the furthest downfield cross-peaks near 132 ppm are in the range of $\omega-3$ DHA sites (*19*). The remaining $-\underline{C}H=$ cross-peaks are selective; they occur only between some sets of the $-\underline{C}H=$ groups. Assignment of $-\underline{C}H=$ frequencies to lipid chemical structures less polyunsaturated than DHA was not attempted. Additional NMR work would be required to assign the additional $-\underline{C}H=$ frequencies to the specific sites on one or more FA chains within one or more lipid structures. The 1D experiments (**Figures 1** and **2**) found that for fish oil and cod extract, the largest NOE increase is at the $-\underline{C}H_3$ peaks near 13 ppm and includes the $\omega-3$ DHA $-\underline{C}H=$ peak near 132 ppm. Thus, at an optimum mixing time, magnetization builds at these two sites and then is transferred via magnetic site exchange to chemically different (magnetically nonequivalent) FA chains. Because polarization transfer in the 2D experiments must always be from sites that relax more slowly to sites that in the same time period had relaxed more quickly, polarization originating in the $-\underline{C}H=$ region from $\omega-3$ DHA must be transferred to other less polyunsaturated lipids. The result was the same in both the fish oil and the cod muscle. This effect from DHA, the most highly polyunsaturated FA in these lipids, is not diluted out by the abundant heterogeneity of the other polyunsaturated lipid structures. Indeed, the heterogeneity may even be required.

The results provide no evidence that the other chain-unique groups (carbonyl, glycerol, phosphocholine) are particularly close in space or that significant magnetic site exchange during long τ_m times is occurring. Thus, extended order in triacylglycerols and phospholipids depends primarily upon the uniform alignment of the lipid hydrocarbon chains and not (as in membranes) on the alignment of hydrophilic headgroups. In PUFA, molecular forces that determine the alignment among the more highly unsaturated double bonds in the $-\underline{C}H=$ regions among FA chains may define the conformation/order within heterogeneous lipids. Additional research is required to differentiate between intra- and inter-FA chain order in heterogeneous lipids.

NOE effects detected by NMR experiments identify inter- and intramolecular sites that are the least mobile in the midst of other sites, which are more highly mobile. Solvents disrupt this inter- and intramolecular order. Less saturated lipids are more rigid at the molecular level with increasing degrees of unsaturation, because double bonds are always more rigid (less mobile) than single bonds. That the more highly unsaturated (and thus the more rigid) lipids are less viscous and have a lower melting point is clearly not yet understood at the molecular level. With these NMR techniques, compounds of known chemical structures can be added to/mixed with natural lipids to alter these NOE effects at the molecular level. The extent to which any added chemical structure including lipoproteins then enhances or interferes with any specific lipid packing is thus open to experimental investigation. Extension of the technique to other natural lipids could prove to be useful to precisely identify the conformation of the particular FA chain(s) within a natural lipid matrix responsible for a specific biochemical purpose. Investigation of DHA from brain or retina lipids, for example, could help to develop molecular site requirements for biophysical or biochemical functionality.

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